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Usefulness of the index of NASH – ION for the diagnosis of steatohepatitis in patients with Non-Alcoholic Fatty Liver: an external validation study.

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List of abbreviations: Non Alcoholic Steato-Hepatitis (NASH), Non Alcoholic Fatty Liver Disease (NAFLD), Index of NASH (ION), Cytokeratin-18 (CK-18), Fibrosis-4 (FIB-4), NAFLD Fibrosis Score (NFS), Receiver Operating Characteristic (ROC), Area Under an ROC Curve (AUROC),

positive predictive value (PPV), negative predictive value (NPV), Metabolic Syndrome (MetS), Type 2 Diabetes Mellitus (T2DM), Liver Stiffness (LS), European Association for the Study of the Liver (EASL), European Association for the Study of Obesity (EASO), European Association for the Study of Diabetes (EASD), Waist Circumference (WC), World Health Organization (WHO), alanine aminotransferases (ALT), aspartate aminotransferases (AST), gamma-glutamyl transpeptidase (GGT), Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), Body Mass Index (BMI), high density lipoprotein (HDL), enzyme-linked immunosorbent assay (ELISA), Standard Deviation (SD), sensitivity (Se), specificity (Sp), positive likelihood ratio (LR+), negative likelihood ratio (LR-), patatin-like phospholipase domain-containing protein 3 (PNPLA3), Area Under the Curve (AUC).

Conflict of interest: no conflict of interest by authors for this study

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Abstract

BACKGROUND & AIMS: The non-invasive identification of steatohepatitis (NASH) in patients with Non-Alcoholic Fatty Liver Disease is an unmet need in clinical practice. Index of NASH (ION) is a new tool for the prediction of NASH. We aimed to externally validate ION and to compare it with CK-18. Since necroinflammation precedes fibrosis, we also tested ION in combination with non-invasive tools for fibrosis.

METHODS: We analyzed data from 292 Italian patients (169 Southern cohort, and 123 Northern cohort) with an histological diagnosis of NAFLD. The ION, FIB-4 and NFS scores were calculated according to published algorithms. Serum cytokeratin18-Aspartate396 levels and liver stiffness (LS) by Fibroscan were assessed within three months from liver biopsy.

RESULTS: The diagnostic accuracy of ION for the identification of NASH was not as satisfactory as reported (area under the ROC curve, AUROC=0.687 [95% CI = 0.62-0.75]). The proposed cut-off value ≥ 50 showed a poor sensitivity (Se) (28%) and a good specificity (Sp) (92%), with a positive predictive value (PPV) of 91% and a negative predictive value (NPV) of 30%. A new cut-off value >26 improved Se (73%) but decreased Sp (60%) (PPV of 84% and a NPV of 43%). ION performed slightly better in obese NAFLD (AUROC= 0.700). The combination of ION and markers of fibrosis did not improve the identification of advanced liver disease.

CONCLUSIONS: ION is not feasible for the non-invasive diagnosis of NASH across different populations of NAFLD patients, mainly because its limited reproducibility in non-obese subjects.

Word count: 244

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- The non-invasive diagnosis of nonalcoholic steatohepatitis (NASH) in patients with nonalcoholic fatty liver disease (NAFLD) is one of the most important unmet needs in clinical practice.
- A new non-invasive algorithm for the prediction of NASH, the ION index, has been recently developed, showing a good diagnostic accuracy in a population of obese NAFLD subjects.

WHAT IS NEW HERE

- ION is not feasible for the non-invasive diagnosis of NASH across different populations of NAFLD patients, mainly because its limited reproducibility in non-obese subjects.
- The combination of ION with a marker of fibrosis does not improve the diagnostic performance of the tools for the non-invasive exclusion of severe fibrosis.

Introduction

Nonalcoholic fatty liver disease (NAFLD), traditionally considered as the hepatic manifestation of the metabolic syndrome (MetS), is rapidly becoming the most common chronic hepatitis in Western countries (1,2) in parallel with the pandemic of obesity and Type 2 Diabetes (T2DM).

NAFLD is a slowly progressive disease, but in clinical practice the early recognition of its potentially progressive phenotype, nonalcoholic steatohepatitis (NASH), as well as the identification of fibrosis, is still elusive (3). Overall, a fair accuracy has been reached in excluding severe (F3/F4) fibrosis by non-invasive markers derived from algorithms such as FIB-4 and NAFLD fibrosis score (NFS) (4-8), or by Liver Stiffness (LS) (9-14), currently suggested as screening tools by the new EASL-EASO-EASD clinical practice guidelines (2). Nevertheless, despite the large effort of the scientific community, to date liver biopsy remains the only procedure able to identify NASH (15-17), but its use for large-scale diagnosis is impracticable. Cytokeratin-18 fragments (CK-18), which are generated during cell death (M65 fragments) or apoptosis (M30 fragments), have a modest accuracy as marker of NASH (18,19). Recently Otgonsuren et al (20) have developed a new algorithm based on the combination of visceral obesity, triglycerides, ALT and HOMA-IR, the index of NASH (ION), able to identify NASH with a good diagnostic accuracy (AUC = 0.88 [95%CI 0.82–0.95]) in a validation cohort with biopsy-proven NAFLD.

The first aims of our study was the external validation of the diagnostic accuracy of the ION index in a cohort of patients with biopsy-proven NAFLD and the comparison of its diagnostic performance with CK-18. Secondly, since the histological features of NASH are often associated with fibrosis or can predict its development, we tested the performance of ION and CK-18 as markers of fibrosis in comparison or in combination with the non-invasive tools currently recommended by guidelines, i.e. FIB-4, NFS and LS.

Material and Methods.

Study population

The study cohort includes a total of 292 Caucasian patients with biopsy proven NAFLD prospectively enrolled in the Division of Gastroenterology of the Torino University Hospital (Northern cohort, N=123) and in the Gastrointestinal and Liver Unit of the Palermo University Hospital (Southern cohort, N=169). The liver biopsy was performed to confirm the diagnosis of NAFLD in patients with features of MetS and ultrasound finding of fatty liver or persistent (> 6 months) elevation of ALT or AST after exclusion of liver disease of other etiology, such as alcohol-induced or drug-induced liver disease, autoimmune or viral hepatitis, and cholestatic or genetic liver disease. All patients had current and past consumption of ethanol less than 20 g per day on direct questioning of both the patients and a close relative. The final diagnosis was based on a liver biopsy showing features consistent with NAFLD.

The study was approved by the ethics committees of the University Hospitals of Torino and Palermo in accordance with the Helsinki Declaration and an informed consent has been obtained by all patients.

Clinical, anthropometric and laboratory data

Clinical and anthropometric data were collected at the time of liver biopsy. Anthropometric indexes (height, weight, WC, and hip circumference) were measured as recommended by the WHO (21). According to their BMI, patients were classified as normal-weight ($\text{BMI} < 24.9 \text{ kg/m}^2$), overweight ($24.9\text{--}29.9 \text{ kg/m}^2$) or obese ($\geq 30 \text{ kg/m}^2$). Laboratory evaluation included liver routine biochemistry (alanine aminotransferases [ALT], aspartate aminotransferases [AST], gamma-glutamyl transpeptidase [GGT] total bilirubin, albumin, platelet count) and metabolic parameters (glucose, insulin, total cholesterol, high density lipoprotein [HDL] cholesterol and triglycerides).

The metabolic syndrome was defined according to the presence of at least three out of five components among waist circumference >94 cm for men and >80 for women, fasting blood glucose > 100mg/dL, triglycerides > 150 mg/dL or under treatment, HDL-cholesterol <40 mg/dL in men, < 50 mg/dL in women, blood pressure >130/85 mmHg or under treatment (25). The degree of insulin

resistance (IR) was derived from the HOMA-IR using the following formula: $\text{insulin} \times \text{glucose} / 22.5$ (26). Blood samples were collected at the time of liver biopsy and stored at -80°C for further analysis.

Non-invasive prediction of NASH and fibrosis

The newly released Index of NASH (ION) was calculated according to the following equation: $1.33 \times \text{waist to hip ratio} + 0.03 \times \text{triglycerides (mg/dL)} + 0.18 \times \text{ALT (U/L)} + 8.53 \times \text{HOMA} - 13.93$ in men; $0.02 \times \text{triglycerides (mg/dL)} + 0.24 \times \text{ALT (U/L)} + 9.61 \times \text{HOMA} - 13.99$ in women (20).

CK18-Asp396 apoptotic fragment determination was assessed by the commercial M30-Apoptosense ELISA Kit (PEVIVA, Sweden) as previously described (22). Briefly, samples were placed into wells coated with mouse monoclonal antibody as a catcher. After washing, a horseradish peroxidase-conjugated antibody (M30) was used for detection. The absorption was determined with an ELISA reader at 450 nm using the cubic spline algorithm. Inter and intra-assay coefficient of variation were below 10%. The concentration of CK18-Asp396 was expressed as units per liter (U/L).

The NAFLD fibrosis score and FIB-4 were calculated from published algorithms (4-8).

Liver stiffness was measured by FibroScan (Echosens, Paris, France) within three months from liver biopsy by an expert operator and was expressed in kilopascals (kPa) as the median value of the successful determinations. Only LS values with at least 10 successful measurements (success rate higher than 60 % and interquartile range minor than 30) were considered reliable and used for the analysis.

Liver Histology

Liver biopsies were stained with hematoxylin and eosin, Masson's trichrome, and special stains for iron and copper. Specimens were analyzed at each clinical center by expert liver pathologists blinded to patient clinical characteristics. The average size of liver biopsies was 25 mm (range 15-

45) with at least 11 portal tracts. Liver biopsies were graded and staged according to Kleiner et al. (15,16). The diagnosis of NASH was done according to the joint presence of steatosis, ballooning and lobular inflammation with or without fibrosis. Severe fibrosis was defined according to the presence of F3 or F4 staging.

Statistical analysis.

Data are reported as mean and standard deviation (SD) for continuous normally distributed variables, as median and 95% confidence interval for the median (95% CI) for continuous non-normally distributed variables and as frequency (%) for categorical variables. Comparisons between groups were performed using the Kruskal-Wallis non-parametric test. For categorical data, the Fisher exact test or the Chi-square test were used as appropriate.

The diagnostic accuracy of ION and CK18-Asp396 apoptotic fragment for the diagnosis of NASH was derived from the Receiver Operating Characteristic (ROC) Curves according to the cut-off values previously described (50 and 279 U/L, respectively) (23). The same analysis was used to assess the ability of each non-invasive score and their combinations to exclude severe fibrosis (F3/F4). We also assessed for the first time the diagnostic accuracy of ION and CK18-Asp396 apoptotic fragment for the prediction of fibrosis. The best cut-offs were derived from the Youden index. Cut-off values for LS, NFS and Fib4 were derived from the literature (4,6,24). Sensitivity (Se), specificity (Sp), positive likelihood ratio (LR+), negative likelihood ratio (LR-), positive predictive value (PPV), negative predictive value (NPV) and the rate of correctly identified cases were calculated accordingly for each index or combination. Values of $p < 0.05$ were considered statistically significant. All the analysis were performed with MedCalc Software version 12 (Mariakerke, Belgium).

Results

Clinical, biochemical and histological features of the study subjects

The anthropometric, biochemical and histological data of the study subjects are reported in Table 1. Overall, MetS was diagnosed in approximately 32% and T2DM in 20% of the patients enrolled. When grouped according to center, visceral obesity was more prevalent in the Southern cohort (73.4% vs 67.5%, $p=0.0102$, respectively) while hypertension in the Northern one (23.7 vs 35%, $p=0.0267$). In the Northern cohort a larger proportion of subjects were lean (21.1% vs 11.8% in the Southern cohort), but the prevalence of the *PNPLA3* rs738409 C>G polymorphism was similar (*PNPLA3* CG 48.9% vs 49.4% and *PNPLA3* GG 23.3% vs 21.3% in the Northern vs Southern cohort, respectively: $p=ns$ for both). In the whole cohort, obesity was found in 40% of cases, half than in the original validation cohort of the ION index, where 95% of subjects were obese. When we subdivided the study subjects according to BMI $<$ or ≥ 30 (Table 2), liver function tests were similar in the two groups but obese patients had a more severe liver damage, including a higher prevalence of NASH. All the non-invasive biomarkers, including ION, and LS were significantly higher in obese compared to non-obese subjects.

Non-invasive prediction of Non-Alcoholic Steatohepatitis

The average value of ION and of serum CK18-Asp396 apoptotic fragment levels in patients with Simple fatty Liver (SFL) and NASH are reported in Figure 1. Both ION and CK18-Asp396 were significantly higher in patients with histological diagnosis of NASH, although the broad range of CK18-Asp396 values confirmed its limited clinical usefulness. In our NAFLD population the diagnostic accuracy of ION for the identification of NASH was less satisfactory than what reported by Otgonsuren et al (20), with an AUROC = 0.687 (95% CI = 0.62-0.75). While in the original validation cohort an ION cut-off ≥ 50 used to identify NASH from simple steatosis provided a sensitivity of 92 and a specificity of 60 (20), in our population the same cut-off had a poor sensitivity (28%) and a good specificity (92%), with a PPV of 91% and a NPV of 30%. Since ION was tested in morbid obese patients, we calculated a new cut-off value for our cohort by the Youden Index. Overall, the new ION cut-off (>26) significantly improved the sensitivity (73%) but

decreased the specificity (60%) for the diagnosis of NASH, with a PPV of 84% and a NPV of 43%, 74% of cases correctly classified (Table 3) and 17.8% of false negative cases. In order to understand how ION is influenced by obesity, we evaluated its diagnostic performance according to BMI (< or ≥ 30). The AUROCs in the two subgroups did not differ significantly, although it was slightly better in the obese one (Table 3). In the obese sub-group, a cut-off > 28 correctly classified 81.5% of cases with a PPV of 88%, a NPV of 36% and 17% of false negative, while in non-obese subjects a cut-off > 29 was able to properly identify 69.9% of cases, with a PPV of 84%, a NPV of 43% and 13.8% of false negative cases (Table 3).

The diagnostic accuracy of CK18-Asp396 for the identification of NASH in our NAFLD population was limited (AUC = 0.60, 95% CI = 0.53-0.66). Overall, the published cut-off value of 279 U/L provided a sensitivity of 48% and a specificity of 68% with a PPV and a NPV of 83% and 29%, respectively. The performance of CK18-Asp396 was unaffected by BMI (data not shown).

At multivariable logistic regression analysis, after controlling for BMI, age, gender and center, ION was the only independent predictor of NASH (Table 4). However, when we re-analysed according to the presence/absence of obesity (BMI ≥ 30), ION was able to predict NASH in the obese sub-group only (OR=4.8, 95%CI=1.1-21.1, p=0.0398; data not shown).

Non-invasive exclusion of severe fibrosis

Since the histological features of NASH are often associated with fibrosis and can predict it, we tested whether both ION and CK18-Asp396 were able to identify the presence and extent of fibrosis. The median values of ION and CK18-Asp396 in the whole population according to staging are shown in Fig.2. ION and CK18-Asp396 significantly increased according to the degree of hepatic fibrosis (Kruskal Wallis p=0.001 and p<0.0001, respectively) and were able to discriminate F0/1 from F2 and F3/F4 (Fig. 2). However, the overall performance of both indexes for the exclusion of severe fibrosis (F3/F4) was poor (AUC=0.61 for ION and AUC=0.67 for CK18-Asp396, Supplementary Figure 1). The newly identified cut-offs > 49 for ION and > 283 U/L for

CK18-Asp396 correctly classified 76% and 74% of cases in the whole cohort. The subdivision according to BMI ($<$ or ≥ 30) did not improve the results (data not shown). The performance of ION and CK18-Asp396 as markers of severe fibrosis was worse compared to LS, NFS and Fib4 (Supplementary Table 1). Among all the non-invasive markers of fibrosis, only Liver stiffness reached an acceptable diagnostic accuracy, defined by $AUC \geq 0.80$, in our NAFLD cohort.

Finally, we tested whether the combination of markers of NASH and fibrosis performed better than two or more markers of fibrosis. The combination of LS and Fib-4 yielded the best results (AUROC 0.878), while LS and ION or LS and NFS had a similar performance. (Supplementary Table 1).

Discussion

The non-invasive identification of NAFLD patients at high risk for advanced liver disease is the most important unmet need in clinical practice: it impedes the identification of subjects who may benefit from more intensive management and closer follow up and it significantly hampers the development of new drugs, currently based on the demonstration of an histological improvement.

Given the close relationship between the progression of the metabolic disturbances and the hepatic damage, several non-invasive algorithms are based on clinical and biochemical components of the MetS. One of the newly released index for the prediction of NASH is ION, based on the combination of visceral obesity, triglycerides, ALT and HOMA-IR. Despite the initially promising results (20), in this study ION did not show an acceptable diagnostic accuracy for the diagnosis of NASH in an external cohort of patients with biopsy-proven NAFLD. ION has been originally validated in a population of morbid obese subjects, hence one of the reasons behind the failure can be related to the different phenotype of NAFLD in our cohort, where 60% of patients were non-obese. The validation of ION in the obese subgroup was slightly, although not significantly, better than in the non-obese one, thus suggesting that ION cannot be considered an universal index and its reliability changes according to the phenotypic expression of NAFLD. In fact, although obesity and

Type 2 Diabetes are classical risk factors for NASH, patients with NAFLD can have different combinations of MetS components with different clinical implications and prognosis: up to one third of our patients had arterial hypertension, which has been linked to a more severe progression of the disease (27).

Another possible bias is the use of ION in patients with Type 2 Diabetes, since the use of HOMA-IR is not recommended in T2DM. ION has been developed in the general population where prevalence of T2DM was around 5% in NAFLD and 0.7% in non-NAFLD (20), while in our cohort T2DM was present in 20% of subject. However, when we repeated the analysis without diabetic subjects, no difference was observed in any of the calculated parameters (data not shown).

Our analysis further confirmed the scarce clinical usefulness of CK18, mainly due to the broad range of values, and highlights once again that up to date there is no reliable marker able to identify the presence of NASH.

Since necroinflammation usually precedes and predicts fibrosis, we also assessed the suitability of the ION index as well as of CK-18 for the non-invasive detection of severe fibrosis. Once again ION performed better than CK-18 in excluding severe fibrosis, but the overall accuracy of ION and CK-18 was modest compared to that of the currently recommended tools (NFS, FIB-4 and LS) and did not provide any significant advantage. The combination of markers of NASH and fibrosis was not better than the combination of two or more markers of fibrosis. The diagnostic accuracy for excluding severe fibrosis was significantly improved by the association of FIB-4 and LS, while the combination of LS with ION or NFS was similar and less satisfactory.

Another important observation stemming from this study is the lower performance of markers based on measures of obesity in non-obese subjects with NAFLD. In particular, the need of a different approach to lean patients with NAFLD is one of the most important issue in clinical practice, but a discussion about the modality is beyond the scope of this study.

This study has some limitations. First of all, patients enrolled at tertiary care centers could be different from the majority of NAFLD/NASH cases in the general population. For the same reasons,

the cut-offs we found and used to evaluate ION could be limited by the validity of the results in different populations and settings. Further, the accuracy of liver biopsy as gold standard to diagnose NASH and fibrosis can be affected by several well-known bias, including sampling errors and interobserver variability. As in other studies, we collected specimens of at least 15 mm of length and the minimum of ten complete portal tracts in order to minimize some risks.

In conclusion, our study showed that ION is not feasible for the non-invasive diagnosis of NASH across different populations of NAFLD patients, mainly because its limited reproducibility in non-obese subjects. The combination of ION with a marker of fibrosis does not significantly improve the diagnostic performance of the tools currently recommended (LS, FIB-4 and LS). Although the identification of mild and moderate fibrosis remains elusive, LS is still the best non-invasive technique for the exclusion of severe fibrosis and its diagnostic performance can be increased by the combination with FIB-4.

References

1. Vernon G, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Aliment Pharmacol Ther* 2011;34:274–285
2. European Association for the Study of the Liver (EASL). Electronic address: easloffice@easloffice.eu; European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO). EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. *J Hepatol*. 2016 Jun;64(6):1388-402. doi: 10.1016/j.jhep.2015.11.004. Epub 2016 Apr 7.
3. Angulo P. Long-term mortality in nonalcoholic fatty liver disease: is liver histology of any prognostic significance? *Hepatology* 2010; 51: 373–5.
4. McPherson S, Stewart SF, Henderson E, et al. Simple noninvasive fibrosis scoring systems can reliably exclude advanced fibrosis in patients with non-alcoholic fatty liver disease. *Gut* 2010; 59: 1265–9.
5. Shah AG, Lydecker A, Murray K, et al. Comparison of noninvasive markers of fibrosis in patients with nonalcoholic fatty liver disease. *Clin Gastroenterol Hepatol* 2009; 7:1104–12.
6. Angulo P, Hui JM, Marchesini G, et al. The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. *Hepatology* 2007; 45:846–54.
7. Cales P, Laine F, Boursier J, et al. Comparison of blood tests for liver fibrosis specific or not to NAFLD. *J Hepatol* 2009; 50: 165–73.
8. Harrison SA, Oliver D, Arnold HL, et al. Development and validation of a simple NAFLD clinical scoring system for identifying patients without advanced disease. *Gut* 2008; 57: 1441–7.
9. Friedrich-Rust M, Ong MF, Martens S, et al. Performance of transient elastography for the staging of liver fibrosis: a meta-analysis. *Gastroenterology* 2008; 134:960–74.
10. Wong VW, Vergniol J, Wong GL, et al. Diagnosis of fibrosis and cirrhosis using liver stiffness measurement in nonalcoholic fatty liver disease. *Hepatology* 2010; 51: 454–62.
11. Petta S, Di Marco V, Camm a C, et al. Reliability of liver stiffness measurement in non-alcoholic fatty liver disease: the effects of body mass index. *Aliment Pharmacol Ther* 2011; 33: 1350–60.
12. Gaia S, Carenzi S, Barilli AL, et al. Reliability of transient elastography for the detection of fibrosis in non-alcoholic fatty liver disease and chronic viral hepatitis. *J Hepatol* 2011; 54: 64–71.

13. Kohlhaas A, Durango E, Millonig G, et al. Transient elastography with the XL probe rapidly identifies patients with nonhepatic ascites. *Hepat Med* 2012; 1: 11–8.
14. Myers RP, Pomier-Layrargues G, Kirsch R, et al. Feasibility and diagnostic performance of the FibroScan XL probe for liver stiffness measurement in overweight and obese patients. *Hepatology* 2012; 55: 199–208.
15. Kleiner DE, Brunt EM. Nonalcoholic fatty liver disease: pathologic patterns and biopsy evaluation in clinical research. *Semin Liver Dis* 2012;32:3–13.
16. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005;41:1313–1321.
17. Bedossa P FLIP Pathology Consortium. Utility and appropriateness of the fatty liver inhibition of progression (FLIP) algorithm and steatosis, activity and fibrosis (SAF) score in the evaluation of biopsies of nonalcoholic fatty liver disease. *Hepatology* 2014;60:565–575.
18. Cusi K, Chang Z, Harrison S, et al. Limited value of plasma cytokeratin-18 as a biomarker for NASH and fibrosis in patients with non-alcoholic fatty liver disease. *J Hepatol* 2014;60:167-174.
19. Kwok R, Tse YK, Wong GL, et al. Systematic review with meta-analysis: non-invasive assessment of non-alcoholic fatty liver disease-the role of transient elastography and plasma cytokeratin-18 fragments. *Aliment Pharmacol Ther* 2014;39:254-269.
20. Otgonsuren M, Estep MJ, Hossain N, Younossi E, Frost S, Henry L, Hunt S, Fang Y, Goodman Z, Younossi ZM. A single non-invasive model to diagnose non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH); *J Gastroenterol Hepatol*. 2014 Dec;29(12):2006-13. doi: 10.1111/jgh.12665
21. WHO Expert Committee. Physical status: the use and interpretation of anthropometry. WHO Library Cataloguing in Publication Data. Geneva, CH, 1995, pp1-452.
22. Rosso C, Caviglia GP, Abate ML, Vanni E, Mezzabotta L, Touscoz GA, Olivero A, Marengo A, Rizzetto M, Bugianesi E, Smedile A. Cytokeratin 18-Aspartate396 apoptotic fragment for fibrosis detection in patients with non-alcoholic fatty liver disease and chronic viral hepatitis. *Dig Liver Dis*. 2016 Jan;48(1):55-61. doi: 10.1016/j.dld.2015.09.008. Epub 2015 Sep 28.
23. Feldstein AE, Wieckowska A, Lopez AR, Liu YC, Zein NN, McCullough AJ. Cytokeratin-18 fragment levels as noninvasive biomarkers for nonalcoholic steatohepatitis: a multicenter validation study. *Hepatology*. 2009 Oct;50(4):1072-8. doi: 10.1002/hep.23050.
24. Wong VW, Vergniol J, Wong GL, Foucher J, Chan HL, Le Bail B, Choi PC, Kowo M, Chan AW, Merrouche W, Sung JJ, de Lédinghen V. Diagnosis of fibrosis and cirrhosis

using liver stiffness measurement in nonalcoholic fatty liver disease. *Hepatology*. 2010 Feb;51(2):454-62. doi: 10.1002/hep.23312.

25. Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *JAMA*. 2001 May 16;285(19):2486-97.
26. Matthews DR, Hosker JP, Rudenski AS et al. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. 1985. *Diabetologia* 28:412–419
27. McPherson S, Hardy T, Henderson E, et al. Evidence of NAFLD progression from steatosis to fibrosing-steatohepatitis using paired biopsies: implications for prognosis and clinical management. *J Hepatol*. 2015;62:1148-55.

Figure legends

Fig. 1. Index Of NASH values (A) and cytokeratin 18-Asp396 apoptotic fragment levels (B) according to the diagnosis of NASH.

Fig. 2. Index of NASH values (A) and cytokeratin 18-Asp396 apoptotic fragment (B) according to the degree of hepatic fibrosis.

Supplementary Fig. 1. Receiver Operating Characteristic (ROC) curves for the identification of severe fibrosis of Index of NASH (A) and cytokeratin 18-Asp396 apoptotic fragment (B).

Table 1. Clinical, biochemical and histological characteristics of the patients population according to the enrollment centre (Southern cohort, N=169 and Northern cohort, N=223).

	Total (N=292)	Southern cohort (N=169)	Northern cohort (N=123)	<i>P</i>
Age, years	44.7 ± 12.8	45.3 ± 13.4	44.5 ± 12.4	0.6187
BMI, kg/m ²	28.9 ± 4.1	29.3 ± 4.1	28.4 ± 3.9	0.0448
Sex, M/F (%)	201/91 (68.8/31.2)	113/56 (66.9/33.1)	88/35 (71.5/28.5)	0.4686
Normal weight /overweight/obese, n (%)	46/130/116 (15.8/44.5/39.7)	20/79/70 (11.8/46.8/41.4)	26/51/46 (21.1/41.5/37.4)	
Waist, cm	99 ± 11	100 ± 12	97 ± 10	0.0347
Hip, cm	104 ± 8	103 ± 8	105 ± 8	0.0220
MS, n (%)	93 (31.8)	50 (29.6)	43 (35)	0.7315
T2DM, n (%)	57 (19.5)	32 (18.9)	25 (20.3)	0.9725
AST, IU/L	36 (35-38)	36 (35-40)	36 (33-40)	0.4578
ALT, IU/L	66 (61-71)	67 (59-73)	64 (56-73)	0.4083
gGT, IU/L	61 (53-68)	58 (49-68)	65 (54-78)	0.3445
BT, mg/dL	0.77 ± 0.72	0.68 ± 0.03	0.91 ± 1.02	0.0197
PLT, 10 ⁹ /L	219 (213-227)	217 (209-228)	222 (213-232)	0.6040
Fasting glucose, mg/dL	93 (90-95)	93 (89-96)	93 (90-96)	0.2779
Fasting insulin, mIU/mL	12.8 (12-14)	14 (12.8-15)	12 (10.3-12.7)	0.0292
HOMA-IR	2.9 (2.75-3.11)	3.1 (2.8-3.5)	2.7 (2.3-3)	0.0337
Total-cholesterol, mg/dL	203 (196-207)	203 (196-211)	200 (184-207)	0.1769
HDL-cholesterol, mg/dL	48 (46-49)	47 (45-50)	48 (46-50)	0.9741
TG, mg/dL	129 (121-140)	133 (122-146)	122 (112-144)	0.7690
LS, kPa	7.1 (6.5-7.7)	6.8 (6.1-7.6)	7.6 (6.6-8.5)	0.0981
CK18-Asp396, U/L	235 (217-255)	252 (242-303)	198 (173-228)	0.0006
ION	32 (29-34)	32 (29-37)	30 (27-35)	0.1508
NFS	-2.341 ± 1.552	-2.296 ± 1.552	-2.406 ± 1.556	0.7984
Fib4	0.89 (0.84-0.96)	0.92 (0.85-1.04)	0.86 (0.8-0.96)	0.2970
Histological features				
Steatosis, n (%)				0.0004
1	126 (43.2)	59 (34.9)	67 (54.5)	
2	95 (32.5)	60 (35.5)	35 (28.4)	
3	71 (24.3)	50 (29.6)	21 (17.1)	
Ballooning, n (%)				0.0020
0	34 (11.6)	13 (7.7)	21 (17.1)	

1	124 (42.5)	69 (40.8)	55 (44.7)	
2	134 (45.9)	87 (51.5)	47 (38.2)	
Lobular inflammation, n (%)				<0.0001
0	40 (13.7)	12 (7.1)	28 (22.8)	
1	165 (56.5)	83 (49.1)	82 (66.7)	
2	80 (27.4)	68 (40.2)	12 (9.7)	
3	7 (2.4)	6 (3.6)	1 (0.8)	
NAS, n (%)				<0.0001
1-2	38 (13)	17 (10)	21 (17.1)	
3-4	122 (41.8)	50 (29.6)	72 (58.5)	
≥ 5	132 (45.2)	102 (60.4)	30 (24.4)	
Fibrosis, n (%)				0.9303
0	77 (26.4)	37 (21.9)	40 (32.5)	
1	67 (22.9)	45 (26.6)	22 (17.9)	
2	75 (25.7)	52 (30.8)	23 (18.7)	
3	53 (18.2)	26 (15.4)	27 (22)	
4	20 (6.8)	9 (5.3)	11 (8.9)	
NASH, n (%)	224 (76.7)	146 (86.4)	78 (63.4)	<0.0001

Data are reported as mean \pm standard deviation for normal continues variables, as median (95% confidence interval for the median) for continues non-normal variables and as frequency (%) for categorical variables.

ALT, aspartate aminotransferases; AST, aspartate aminotransferases; BMI, body mass index; BT, total bilirubin; CK18-Asp396, cytokeratin 18-Aspartate 396 apoptotic fragment; gGT, gamma-glutamyl aminotransferases; HDL, high density lipoprotein; HOMA, homeostasis model of assessment; ION, index of non-alcoholic steatohepatitis; IR, insulin resistance; LS, liver stiffness; MS, metabolic syndrome; NASH, non-alcoholic steatohepatitis; NFS, non-alcoholic fatty liver disease fibrosis score; PLT, platelets; TG, triglycerides; T2DM, type 2 diabetes mellitus.

Table 2. Clinical, biochemical and histological characteristics of the patients population according to the presence of obesity (OB) (Non-Obese, N=176 and Obese, N=116).

	Non-OB (N=176)	OB (N=116)	P
Age, years	43.7 ± 13.1	46.9 ± 12.6	0.0378
Sex, M/F (%)	127/49 (72.2/27.8)	74/42 (63.8/36.2)	0.1672
AST, IU/L	36 (33-38)	38 (35-43)	0.0662
ALT, IU/L	69 (58-73)	65 (59-74)	0.5870
gGT, IU/L	61 (49-75)	61 (54-70)	0.4837
HOMA-IR	3.03 ± 2.42	5.00 ± 3.4	<0.0001
LS, kPa	6.1 (5.9-6.6)	8.8 (8.0-9.9)	<0.0001
CK18-Asp396, U/L	208 (186-243)	266 (235-316)	0.0003
ION	28 (26-32)	35 (32-43)	0.0038
NFS	-2.701 ± 1.370	-1.795 ± 1.655	<0.0001
Fib4	1.06 ± 0.78	1.32 ± 0.92	0.0108
Histological features			
Steatosis, n (%)			0.0002
1	89 (50.6)	37 (31.9)	
2	56 (31.8)	39 (33.6)	
3	31 (17.6)	40 (34.5)	
Ballooning, n (%)			0.0040
0	25 (14.2)	9 (7.8)	
1	82 (46.6)	42 (36.2)	
2	69 (39.2)	65 (56.0)	
Lobular inflammation, n (%)			0.0068
0	31 (17.6)	9 (7.7)	
1	101 (57.4)	64 (55.2)	
2	40 (22.7)	40 (34.5)	
3	4 (2.3)	3 (2.6)	
NAS, n (%)			<0.0001
1-2	32 (18.2)	6 (5.2)	
3-4	80 (45.4)	42 (36.2)	
≥ 5	64 (36.4)	68 (58.6)	
Fibrosis, n (%)			<0.0001
0	60 (34.1)	17 (14.7)	
1	48 (27.3)	19 (16.4)	

2	40 (22.7)	35 (30.2)	
3	20 (11.4)	33 (28.4)	
4	8 (4.5)	12 (10.3)	
NASH, n (%)	126 (71.6)	98 (84.5)	0.0160

Data are reported as mean \pm standard deviation for normal continues variables, as median (95% confidence interval for the median) for continues non-normal variables and as frequency (%) for categorical variables.

ALT, aspartate aminotransferases; AST, aspartate aminotransferases; CK18-Asp396, cytokeratin 18-

Aspartate 396 apoptotic fragment; gGT, gamma-glutamyl aminotransferases; HDL, high density lipoprotein;

HOMA, homeostasis model of assessment; ION, index of non-alcoholic steatohepatitis; IR, insulin

resistance; LS, liver stiffness; NASH, non-alcoholic steatohepatitis; NFS, non-alcoholic fatty liver disease

fibrosis score; OB, obese; PLT, platelets.

Table 3. Area under the curve, sensitivity, specificity, positive predictive value, negative predictive value and accuracy of ION for the diagnosis of NASH according to the presence of obesity (OB).

ION	AUC (95% CI)	Cut-off	Se (%)	Sp (%)	PPV (%)	NPV (%)	Cases correctly classified
All subjects	0.687 (0.62-0.75)	> 26	73	60	84	43	74.5 %
Non-OB (BMI < 30 kg/m ²)	0.663 (0.58-0.74)	> 29	57	74	84	43	69.9 %
OB (BMI ≥ 30 kg/m ²)	0.700 (0.59-0.79)	> 28	79	53	88	36	81.5 %

AUC, area under the receiver operating characteristic curve; BMI, body mass index; CI, confidence interval; ION, index of non-alcoholic steatohepatitis; NPV, negative predictive value; OB, obese; PPV, positive predictive value; Se, sensitivity, Sp, specificity.

Table 4. Univariate and multivariable logistic regression analysis of Index of NASH and cytokeratin 18-Asp396 for the diagnosis of NASH.

All subjects (N=292)				
	Univariate analysis		Multivariable analysis	
	OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>
Age, y	1.0 (0.9-1.0)	0.5347	0.99 (0.96-1.0)	0.4100
Sex, M	0.3 (0.2-0.7)	0.0031	0.4 (0.1-0.9)	0.0395
BMI, kg/m ²	1.2 (1.1-1.2)	0.0002	1.2 (1.0-1.3)	0.0080
Southern Center	3.7 (2.1-6.5)	< 0.0001	6.3 (2.7-14.5)	< 0.0001
ION > 26	3.9 (2.1-7.1)	< 0.0001	2.6 (1.2-5.7)	0.0156
CK18-Asp396 ≥ 279	1.6 (0.8-3.1)	0.1447	0.6 (0.3-1.4)	0.2471

BMI, body mass index; CI, confidence interval; CK18-Asp396, cytokeratin 18 aspartate 396 apoptotic fragment; ION, index of non-alcoholic steatohepatitis; M, male gender; OR, odd ratio; y; years